

AD \_\_\_\_\_

Award Number: DAMD17-99-1-9524

TITLE: Targeted Antibody Inhibition of Bone Metastases by  
Prostate Cancer

PRINCIPAL INVESTIGATOR: XiuHua Sun, Ph.D.  
John Chirgwin, Ph.D.

CONTRACTING ORGANIZATION: Univeristy of Texas  
San Antonio, Texas 78284-7828

REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20010124 025

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE  
August 2000

3. REPORT TYPE AND DATES COVERED  
Annual Summary (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE

Targeted Antibody Inhibition of Bone Metastases by Prostate Cancer

5. FUNDING NUMBERS

DAMD17-99-1-9524

6. AUTHOR(s)

John Chirgwin, Ph.D. XiuHua Sun, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas  
San Antonio, Texas 78284-7828

E-MAIL: sunx0@uthscsa.edu

8. PERFORMING ORGANIZATION  
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING  
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Metastasis to bone is a major cause of morbidity from prostate cancer. Parathyroid hormone-related protein (PTHrP) is expressed by prostate cancer cells and stimulates osteoclasts. My project is to test whether an antibody against PTHrP will block prostate cancer metastasis to bone. I cloned the light and heavy chain cDNAs from a hybridoma, which secretes a PTHrP-neutralizing antibody which blocks breast cancer metastasis to bone but is untested against prostate cancer. The cDNAs in a bicistronic expression vector produced mouse IgG when transfected into human 293 cells. Secreted mouse antibody inhibited osteoclast formation and bone resorption in two tissue culture assays stimulated with 20ng/mL PTHrP (1-34) peptide. Next, the DNA will be transfected into the human prostate cancer cell line PC3, which secretes PTHrP and causes osteolytic metastases. PC3 clones which neutralize their own PTHrP will be tested for their ability to metastasize to bone in animals, which is expected to be greatly reduced compared to controls. In the first year of my fellowship I have learned many molecular and immunological techniques. In the second year I will learn many animal techniques related to bone metastases, including quantitative x-ray image analysis and skeletal histomorphometry.

14. SUBJECT TERMS

Prostate Cancer

15. NUMBER OF PAGES

28

16. PRICE CODE

17. SECURITY CLASSIFICATION  
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION  
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION  
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

## Table of Contents

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Table of Contents.....</b>	<b>3</b>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>5-9</b>
<b>Key Research Accomplishments.....</b>	<b>12</b>
<b>Reportable Outcomes.....</b>	<b>12,13</b>
<b>Conclusions.....</b>	<b>9</b>
<b>References.....</b>	<b>10,11</b>
<b>Appendices.....</b>	<b>13-23</b>

## Xiuhua Sun, M.D., Targeted Antibody Therapy to Inhibit Bone Metastases by Prostate Cancer

Army Prostate Cancer Program Postdoctoral Fellowship Progress Report - 01 Year

### Abstract:

Metastasis to bone is a major cause of morbidity and mortality from prostate cancer. Most men dying from the disease have skeletal metastases, which cause abnormal bone growth, resulting in bone pain, fracture, and nerve compression. Prostate cancer prefers to grow in bone, since bone matrix stores large quantities of immobilized growth factors, which can stimulate tumor cells. When cancer cells produce factors which stimulate bone resorption, metastasis to bone is enhanced: tumor cell products stimulate cells called osteoclasts to release growth factors from the bone matrix. These products in turn stimulate the growth of tumor cells. Tumor-secreted factors capable of stimulating bone resorption are thus prime candidates to contribute to the establishment of bone metastasis. Parathyroid hormone-related protein (PTHrP) is known to be expressed by prostate cancer cells and is a major stimulator of osteoclasts. My project is **to test whether an antibody against PTHrP will block prostate cancer metastasis to bone**. I will use a gene expression vector to introduce the inhibitory antibody expression directly into the prostate cancer cells. This will be a **test to target inhibitors directly to the site of metastasis, which could be a useful approach for future gene therapy**.

In the first year of the work proposed I cloned and sequenced the light and heavy chain cDNAs from the 3F5 hybridoma, which secretes a monoclonal antibody directed against the N-terminal region of PTHrP. This antibody blocks breast cancer metastasis to bone but has never been tested against prostate cancer. The two antibody chain cDNAs were transferred into a bicistronic expression vector, producing mouse IgG antibody when the DNA was transiently transfected into human 293 cells in vitro. The secreted antibody potently and completely inhibited osteoclast formation and bone resorption in two tissue culture assays stimulated with 20ng/mL PTHrP (1-34) peptide. The DNA is presently in a vector designed for gene therapy.

In the coming year I will transfer the antibody-coding portion of this DNA into a vector, pcDNA3neo, known to be able stably to transfect cancer lines. This DNA will be transfected into the human prostate cancer cell line PC3, which our laboratory has shown to secrete abundant PTHrP and reliably to cause osteolytic metastasis in an animal model. PC3 cell subclones will be selected which stably neutralize, via the transfected antibody, the PTHrP which they also secrete. These cells will be tested for their ability to metastasize to bone in animals, which is expected to be greatly reduced compared to control PC3 cells. In this experiment I will test three parallel therapeutic approaches to inhibiting bone metastases with PTHrP-neutralizing antibody: 1) conventional systemic injection of purified antibody; 2) intramuscular injection of naked DNA encoding the same antibody (which should drive muscle secretion of the antibody into the systemic circulation); and 3) use of tumor cells secreting their own antibody (which should guarantee high concentrations of neutralizing antibody at the metastatic sites).

In the first year of my fellowship training I have learned many molecular and immunological techniques. I will present my results at the American Society of Bone & Mineral Research in Toronto in September. In the second year of my project I will learn many animal techniques related to bone metastases, including quantitative x-ray image analysis and skeletal histomorphometry.

## Targeted Antibody Therapy to Inhibit Bone Metastases by Prostate Cancer

### Introduction:

Cancer of the prostate is the second leading cause of male deaths from cancer in the United States (Karp et al., 1996). The disease resulted in about 40,000 deaths in 1996. Up to 84% of patients dying of prostate cancer have been found to have bone metastases at autopsy (Jacobs, 1983). The metastases are predominantly osteoblastic, resulting in net gain of bone. The new bone formation is often disorganized and, particularly in the spine, can result in nerve compression syndromes, as well as bone pain. Mean survival from the time of diagnosis of bone involvement is 40 months. The frequency of prostate cancer metastasis to bone and the long duration of the disease result in a major contribution to patient morbidity from osteoblastic metastases (Coleman, 1997). However, the mechanisms responsible for prostatic cancer cell growth at metastatic sites in bone are incompletely understood.

*Role of bone resorption in prostate cancer metastasis.* Serum markers of bone resorption are elevated in prostate cancer metastatic to bone, suggesting that the bone lesions contain a lytic component associated with the blastic lesions. Bone resorption may release a variety of growth-regulatory factors, previously immobilized in the bone matrix, which can in turn stimulate the local growth of tumor cells. Thus, prostatic metastasis to bone may involve ongoing bone turnover, rather than requiring bone resorption only for the initial establishment of the metastatic foci (Goltzmann, 1997). Such a mechanism, linking metastasis by cancer cells with osteolytic bone resorption, has been experimentally established for human breast and lung cancers (Guisse, 1997). The laboratory I am working in has established that PC3 prostate cancer cells cause very similar osteolytic metastases. All of these osteolytic tumor types secrete parathyroid hormone-related protein, PTHrP.

*PTHrP and prostate cancer.* Prostate cancer was first reported to express PTHrP by Iwamura et al. (1993). The human PC3 cell line secretes substantial amounts of PTHrP under serum-free conditions. However, PTHrP appears not to be an autocrine growth factor for normal prostatic cells (Peehl et al., 1997). Signaling through the G protein-coupled PTH/PTHrP receptor is not generally mitogenic (Guisse & Mundy, 1996), but Dougherty et al. (1999) recently demonstrated growth dependence of prostate cancer on intracellular PTHrP. It has also been reported that the expression of PTHrP, assayed immunohistochemically, increased with increasing tumorigenic progression of prostate cells (Asadi et al., 1996). Thus the roles of PTHrP in prostate cancer growth may be complex. The group in which I am training has demonstrated that neutralizing antibodies against PTHrP can effectively decrease bone metastases (Guisse et al., 1996; Yin et al., 1999.) The latter experiments used a monoclonal antibody called 3F5 raised against PTHrP. This antibody plays a central role in my research. It was developed by Dr. V. Grill in Australia and has been tested extensively (Rankin et al., 1995; 1997). Yin et al. (1999) demonstrated that this antibody could effectively block osteolytic metastases caused by human MDA-MB-231 breast cancer cells secreting PTHrP. Because of the slow development of bone metastases in prostate cancer, any treatment, such as administration of a therapeutic neutralizing antibody, would have to be repeated at least weekly over the course of many months. We decided instead to take a gene-therapy-related approach. We undertook to clone the light and heavy chain cDNAs encoding the 3F5 mAb, express both chains from a single DNA vector, and then stably express this DNA in either the animals or the metastatic tumor cells themselves. In the first year of my training I have succeeded in cloning, expressing, and functionally assaying the 3F5 mAb. Stable clones of PC3 prostate cancer cells are

now being prepared which secrete their own PTHrP-neutralizing antibody.

*Recombinant secretion of cloned antibodies.* Functional antibodies can be readily expressed in non-lymphoid cells (Biocca et al., 1990), which permits them to be delivered to specific anatomical sites *in vivo* (Piccioli et al., 1991). In the experiments presented here, the intent was to neutralize the extracellular effects of tumor-produced PTHrP on target cells in the bone microenvironment. This will be much more efficiently achieved by delivering the antibody to the tumor site in bone, rather than injecting animals with sufficiently large amounts of purified antibody to cause systemic neutralization of PTHrP. Alternative approaches to blocking tumor-produced PTHrP, such as anti-sense, could have multiple side effects by inhibiting intracellular versus extracellular functions of PTHrP (Dougherty et al., 1999). Intracrine actions of PTHrP, which may have growth effects on prostate cancer cells, could be mediated by nuclear targeting of intracellular PTHrP (Lam et al., 2000). Antibody secretion directed to the metastatic site should not interfere with confounding intracrine pathways involving PTHrP. Therefore we set out to clone the sequences encoding the 3F5 monoclonal antibody which neutralizes N-terminal PTHrP (Rankin et al., 1995). We have isolated the light and heavy chains of the mAb by PCR of RNA isolated from the hybridoma and primers against regions of conserved immunoglobulin sequence. After sequencing, the two chains were transferred into a bicistronic vector which expresses both chains on a single mRNA, separated by an internal ribosome reentry site (Duke et al., 1992). This permits equimolar biosynthesis of the two chains and avoids cellular toxicity associated with excess production of immunoglobulin heavy chains (Kolb & Siddell, 1997).

#### **Methods:**

RNA was isolated from the 3F5 hybridoma and reverse transcribed into oligo(dT)-primed cDNA using commercial kits (GIBCO-LifeTechnologies) according to the manufacturer's instructions. PCR kits were obtained from the same source. PCR products of the correct sizes were purified from agarose gels with a commercial kit (Qiagen), digested with the appropriate restriction enzymes and repurified by agarose gel electrophoresis. All DNA enzymes were obtained from New England BioLabs and used according to the manufacturer's recommendations. All PCR products were initially subcloned into pBluescript vector (Stratagene) and analyzed by restriction mapping, PCR, and automated DNA sequencing by the Center for Advanced DNA Technologies of this University. Sequence data were analyzed with MacVector 6.5 software (Oxford Molecular). Initial DNAs were ligated into the subcloning vector using polylinker sites in the plasmid corresponding to the restriction sites originally designed into the PCR primers. Ligated DNAs were transformed into competent *E. coli* DH5 $\alpha$  cells (GIBCO-LifeTechnologies) according to the manufacturer's instructions. The light chain was constructed as a 0.7kb *Pst* I to *Sal* I fragment and the heavy chain as a 1.4kb *Xba* I to *Bgl* II fragment. Plasmid inserts with the correct sequence were transferred into the bicistronic expression vector pVR1030, provided by Dr. P.M. Hobart (Vical, Inc, San Diego, CA), which carries a kanamycin resistance gene. The light chain was subcloned into pVR1030, and an initial subclone was isolated and mapped. The heavy chain was then subcloned into this intermediate to give the final plasmid, designated pVR3F5. DNAs were purified with Qiagen kits.

Expression DNA pVR3F5 was transfected into 293 cells (for transient assay) and into human breast MDA-MB-231 and human prostate PC3 cancer cell lines using Lipofectamine Plus kits (GIBCO-LifeTechnologies) according to the manufacturer's instructions. One day after transfection the medium was changed to serum-free, and this medium was conditioned for 48hrs. Cell number was determined, and the media were assayed for mouse IgG content and for their ability to neutralize

the bone-resorbing and osteoclastogenic activities of synthetic human PTHrP 1-34 (R&D Systems). Mouse marrow culture assays were carried out by standard means (Takahashi et al., 1988). Fetal rat long bone organ culture following standard procedures (Garrett et al., 1990) was kindly carried out by Mr. Paul Williams. Concentrations of mouse IgG in the serum-free media conditioned by human cells were determined by a capture ELISA, following standard procedures (Harlow & Lane, 1988). Standard mouse IgG, goat anti-mouse antibody conjugated to alkaline phosphatase, and alkaline phosphate substrate were from Sigma. Stable cell lines were selected by the addition of 500-800µg/ml G418 (GIBCO-LifeTechnologies) 48 hours after transfection with 9 parts pVR3F5 and 1 part pcDNA3 (to provide *neo* resistance).

## Results:

*Isolation of heavy chain clones.* PCR primers were designed according to Coloma et al., 1992). A mixture of three 5' primers and one 3' primer was used. Mixed bases at a single position are indicated by parentheses, and the added *Xba* I and *Bgl* II sites are underlined:

g1 [5' GCTCTAGACACCATGG (AG)ATG(CG)AGCTG(TG)GT(CA)AT(CG)CTCTT 3']

g2 [5' GCTCTAGACACCATG(AG)ACTTCGGG(TC)TGAGCT(TG)GGTTTT 3']

g3 [5' GCTCTAGACACCATGGCTGTCTTGGGGCTGCTCTTCT 3']

g-3' [5' GAAGATCTTCATTTACCAGGAGAGTGGGAGAGGCTCTTCTCAGT 3']

PCR (30 cycles) under standard conditions gave a band of the expected size (1.4kb), which was subcloned into pBluescript and sequenced. The 1411 bp insert was derived from the g2 + g-3' primer pair and encoded a 464 amino acid heavy chain including signal peptide, shown in figure 1C. The DNA sequence includes internal sites for *Pst* I and *Sal* I, necessitating that the heavy chain be transferred into the expression vector only after the light chain had been subcloned via these two restriction sites.

*Isolation of light chain clones.* PCR primers were designed as above. A mixture of four 5' primers and one 3' primer was used. Mixed bases at a single position are indicated by parentheses, and the added *Pst* I and *Sal* I sites are underlined:

k1 [5' AACTGCAGACCATGGAGACAGACACACTCCTGCTAT 3']

k2 [5' AACTGCAGACCATGGATTTTCAAGTGCAGATTTTCAG 3']

k3 [5' AACTGCAGACCATGGAG(TA)CACA(GT)(TA)CTCAGGTCTTT(GA)TA 3']

k4 [5' AACTGCAGACCATG(GT)CCCC(AT)(GA)CTCAG(CT)T(CT)CT(TG)GT 3']

k-3' [5' GACGTCGACCTAACACTCATTCCTGTTGAAGCTCTTGACAATGGG 3']

PCR (30 cycles) under standard conditions gave a band of the expected size (0.75kb), which was subcloned into pBluescript and sequenced. Over a dozen clones were sequenced, all of which encoded a transcript previously reported to be expressed by the MOPC-21 tumor line, which is the partner in the cell fusion step of hybridoma generation (Coloma et al., 1992). This aberrant transcript has a frame shift in the kappa constant region and does not result in light chain protein synthesis. Attempts to reduce the amplification of this transcript by PCR optimization were unsuccessful. When tested pairwise, only the k1 + k-3' primer pair gave a PCR band of the correct size. We designed an aberrant chain-specific PCR primer whose 3' end annealed to the 4 extra bases found in this transcript (Coloma et al., 1992, figure 2). Individual clones of 3F5 cDNA amplified with k1 + k-3' and ligated as .75kb *Pst* I and *Sal* I fragments into pBluescript were isolated. Miniprep DNAs from white colonies on *lac* indicator plates were individually analyzed by PCR with k1 + k-3' for presence of insert and with k1 + aberrant primer. Screening of over 50 colonies yielded two aberrant-negative candidates, which were designated K1 and K10. When sequenced, both encoded a nearly

identical mouse IgG<sub>1</sub> kappa light chain without signal peptide. K1 and K10 differ by three N-terminal amino acid residues, designated 25-27, in Figure 1. The nucleotide sequences for the two cDNAs were identical elsewhere, suggesting that the amino acid differences represented a polymorphism. Near the 5' end of each clone was an *Nco* I restriction site which includes a Met codon (residue 23 in figure 1). The signal peptide of the aberrant V<sub>κ</sub> transcript was isolated by PCR as a 75bp *Xba* I to *Nco* I fragment and ligated into the 5' ends of the K1 and K10 pBluescript subclones digested with the same two enzymes. The final pre-light chain protein sequences are shown in figures 1A and B.

*Construction of pVR3F5.* The light and heavy chains were cloned successively into the *Pst* I and *Sal* I sites and *Xba* I and *Bgl* II sites, respectively, of pVR1030. The map of the bicistronic vector is shown in figure 2. Transcription is driven by a CMV promoter. A major feature of the vector is the inclusion, between the cloning sites for the light and heavy chains, of an internal ribosome reentry sequence of viral origin, CITE, which permits two proteins to be translated in roughly equimolar amounts from a single mRNA transcript (Duke et al., 1992). The use of the bicistronic vector assures that the cloned antibody will be efficiently expressed, since the light and heavy chains should be synthesized in equal amounts. The vector carries a prokaryotic kanamycin resistance cassette but lacks a selectable marker for use in mammalian cells.

*Expression of pVR3F5.* The expression DNAs, in both K1 and K10 versions, were purified and tested for their ability to cause secretion of functional mouse IgG antibody. DNAs, versus control pVR1030, were transiently transfected into the human embryonal kidney cell line HEK 293. Serum-free conditioned media were assayed (figure 3) and found to contain about 50ng/ml mouse IgG. The procedure was repeated on a larger scale in T150 tissue culture flasks, which gave about 100ng/ml mouse IgG (figure 4). Both antibodies were effective, suggesting that the polymorphic differences between K1 and K10 did not affect binding to PTHrP. The K1 and K10 IgGs were concentrated by chromatography on Protein A/G minicolumns (Pierce ImmunoPure (A/G) IgG purification kit) according to the manufacturer's instructions. The concentrations of the purified proteins were determined with the IgG ELISA, and these materials were used in the subsequent assays.

*Antibody produced from pVR3F5 blocks osteoclast formation in vitro.* Osteoclast formation was assayed in cultures of mouse bone marrow (Takahashi et al., 1988) stimulated with either 10<sup>-8</sup>M 1,25di(OH)vitamin D<sub>3</sub> or 20ng/ml PTHrP 1-34, which result in the formation of multinucleated osteoclast-like cells that stain positively for tartrate-resistant acid phosphatase [TRAP, assayed with a Sigma kit]. Figures 5-8 show that the pVR3F5-encoded IgG inhibited the formation of osteoclast-like cells in response to either stimulus. Both K1 and K10 antibodies appeared to be equivalently effective. Inhibition of the response to PTHrP was as expected. We do not have an explanation for the unanticipated inhibition of osteoclast-like cell formation in response to calcitriol.

*Antibody produced from pVR3F5 blocks PTHrP-stimulated bone resorption in vitro.* The addition of either K1 or K10 versions of the pVR3F5-derived antibody completely inhibited Ca<sup>45</sup> release from labeled fetal rat long bones in culture (Figure 9).

*Stable cell lines express 3F5 antibody.* pVR3F5-k1 and pVR3F5-k10 were stably cloned into the human cancer lines PC3 (prostate) and MDA-MB-231 (breast), both of which secrete PTHrP and cause osteolytic metastases in a mouse model (Guise, 1997). Co-selection for G418 resistance resulted in the initial identification of about 20 positive clones out of 100 single-cell clones for each cell line. These secreted up to 40ng/ml mouse IgG per 10<sup>6</sup> cells per 48hrs when first screened. Upon continuous passaging in tissue culture for 8 weeks every clone showed decreasing IgG secretion,



despite continuing resistance to 500µg/ml G418. At the end of two months, no positive expressing clones remained. A parallel experiment selecting for secretion of a protein from a pcDNA3 plasmid construct [which carries G418 resistance and CMV-driven protein expression on the same DNA] showed no such loss of protein expression in either cell line. We concluded that the pVR1030 vector does not confer stable expression.

*Recloning of bicistronic antibody cassette into stable vector.* The light chain + 502bp CITE sequence [1.2kb *Pst* I to *Xba* I fragment] and the heavy chain sequence [1.4kb *Xba* I to *Bgl* II fragment] are presently being subcloned into pBluescript. In the case of the heavy chain subclone, the *Bgl* II sticky end will be ligated (irreversibly) to the *Bam*HI site of the vector polylinker. The two fragments will be released from the pBluescript subclones as *Eco*R I to *Xba* I 1.2kb [pre-light chain + CITE] and *Xba* I to *Apa* I 1.4kb [heavy chain] fragments, which will then be sequentially ligated into the stable expression vector pcDNA3 (Clontech) digested with these same restriction enzymes. The product, pcDNA3F5, will be tested for transient IgG expression, then used to re-generate the stable clones in PC3 and MDA-MB-231 cells, as described above. pcDNA3 carries a *neo* resistance cassette which permits stable selection of mammalian cells with G-418. We plan to use the K10 version for subsequent experiments, for the sake of simplicity, since both K1 and K10 appear to encode equivalent neutralizing activities.

### Summary:

In the first year of the proposal, I have been fully successful. I carried out tasks 1-6 of the total of 10 tasks proposed in the statement of work (p. 5 of the original application). Light and heavy chains of the 3F5 monoclonal antibody were cloned and sequenced and shown to direct efficient synthesis of antibody able to neutralize the actions of PTHrP to stimulate osteoclast formation and bone resorption-which are essential components of osteolytic metastases *in vivo*. My results to date will be presented at the national meeting of the American Society of Bone and Mineral Research in Toronto in late September.

### Plans for Year 2:

Task 5 of the statement of work will be repeated, as described two paragraphs above, to place the sequences necessary for bicistronic expression of the 3F5 IgG light and heavy chains into a vector, pcDNA3, known to give stable clones when introduced into prostate cancer cells. Tasks 6 and 7, generation of stable clones in PC3 cells and determination of growth rates will be carried out. I expect to be ready to carry out the central experiment of the proposal by January 200, task 8, the *in vivo* metastasis experiment by injecting the stable PC3 cells into the left cardiac ventricle of male nude mice. This experiment takes 2-3 months, following which the final 2 tasks, to analyze the metastases in the animals, will be carried out in routine fashion. I am also testing whether it is possible to assay the levels of the 3F5 mAb in the circulation of the tumor-bearing mice. PTHrP1-34 will be used as the antigen to coat wells as the capture reagent for the 3F5 mAb, which will be detected with the goat anti-mouse IgG alkaline phosphatase conjugate described above in Methods. I am currently investigating the possibility of using the already made and tested pVR3F5 DNA as a naked DNA treatment (Restifo et al., 2000) of mice in the metastasis model. Intramuscular injection of naked DNA will direct synthesis of 3F5 mAb into the circulation of the mice. The concentrations could be sufficient to neutralize the osteolytic effects of PC3-secreted PTHrP. I have not yet obtained protocols for the naked DNA treatment experiments; so this strategy is currently only hypothetical, but it could be tested in parallel with the strategies already developed and which

will be tested in the coming year. Naked DNA technology has already entered clinical trials for immunotherapy of prostate cancer (Mincheff et al., 2000).

### **Training Component:**

I have found the training environment in the Division of Endocrinology to be highly useful and stimulating. I attend weekly Divisional data presentation meetings, at which I present my results to the faculty and post-doctoral fellows once a month. I attend a weekly Chirgwin-Guise joint laboratory meeting at which I present my results every week. I also participate in a biweekly bone cell biology journal club and attend a weekly endocrinology seminar program. Dr. Ian Thompson, Chief of Urology, has organized a prostate cancer research program with monthly meetings, which I attend. I participated in the one-day-long San Antonio Cancer Symposium in July and a scientific retreat to Corpus Christi in August of the Metastasis Program of the San Antonio Cancer Institute. In September I will also participate in a 2-day training program in public presentation skills for scientists conducted by Brent Consulting Group and sponsored by my Division and Novartis Pharmaceutical. This will be of particular help to me since English is not my native tongue. Dr Chirgwin's laboratory provides full access in the laboratory to library facilities, e journals and literature databases, such as PubMed, through two computers. The substantial group of postdoctoral fellows interested in prostate cancer in general and bone metastasis in particular in the laboratories of Drs. Guise, Chirgwin, Yoneda, Harris, and Bonewald provides a great deal of intellectual stimulation relevant to my scientific interests in the metastasis of prostate cancer to bone.

### **References:**

- Asadi F, Farraj M, Sharifi R, Malakouti S, Antar S, Kukreja S (1996). Enhanced expression of parathyroid hormone-related protein in prostate cancer as compared with benign prostatic hyperplasia. *Hum Pathol* **27**: 1319-1323.
- Biocca S, Neuberger MS, Cattaneo A (1990). Expression and targeting of intracellular antibodies in mammalian cells. *EMBO J* **9**:101-108.
- Carroll WL, Mendel E, Levy S (1988). Hybridoma fusion cell lines contain an aberrant kappa transcript. *Mol Immunol* **25**:991-995.
- Coleman RE (1997). Skeletal complications of malignancy. *Cancer* **80**: 1588-1594.
- Coloma MJ, Hastings A, Wims LA, Morrison SL (1992). Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. *J Immunol Methods* **152**:89-104.
- Dougherty KM, Blomme EA, Koh AJ, Henderson JE, Pienta KJ, Rosol TJ, McCauley LK (1999). Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res* **59**: 6015-6022.
- Duke GM, Hoffman MA, Palmenberg AC (1992). Sequence and structural elements that contribute to efficient encephalomyocarditis virus RNA translation. *J Virol* **66**:1602-1609.
- Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR (1990). Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J Clin Invest* **85**:632-639.
- Goltzmann D (1997). Mechanisms of the development of osteoblastic metastases. *Cancer* **80**: 1581-1587.
- Guise TA, Mundy GR (1998). Cancer and bone. *Endo Rev* **19**: 18-54.
- Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR (1996).

Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* **98**: 1544-1549.

Guise TA (1997). Parathyroid hormone-related protein and bone metastases. *Cancer* **80**: 1572-1580.

Harlow E, Lane D (1988). Antibodies, A Laboratory Manual. Cold Spring Harbor Press, Cold Spring NY.

Iwamura M, di Sant'Agnese PA, Wu G, Benning CM, Cockett ATK, Deftos LJ, Abrahamsson P-A (1993). Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res* **53**: 1724-1726.

Iwamura M, Wu G, Abrahamsson P-A, Cockett ATK, Foss KA, Deftos LJ (1994). Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. *Urology* **43**: 675-679.

Jacobs SC (1983). Spread of prostatic cancer to bone. *Urology* **21**: 337-344.

Karp JE, Chiarodo A, Brawley O, Kelloff GJ (1996). Prostate cancer prevention: investigational approaches and opportunities. *Cancer Res* **56**: 5547-5556.

Kolb AF, Siddell SG (1997). Expression of a recombinant monoclonal antibody from a bicistronic mRNA. *Hybridoma* **16**:421-426.

Lam MH, Thomas RJ, Martin TJ, Gillespie MT, Jans DA (2000). Nuclear and nucleolar localization of parathyroid hormone-related protein. *Immunol Cell Biol* **78**:395-402.

Mincheff M, Tchakarov S, Zoubak S, Loukinov D, Botev C, Altankova I, Georgiev G, Petrov S, Meryman HT (2000). Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: A phase I/II clinical trial. *Eur Urol* **38**:208-217

Peehl DM, Edgar MG, Cramer SD, Deftos LJ (1997). Parathyroid hormone-related protein is not an autocrine growth factor for normal prostatic epithelial cells. *Prostate* **31**: 47-52.

Piccioli P, Ruberti F, Biocca S, Di Luzio A, Werge TM, Bradbury A, Cattaneo A (1991). Neuroantibodies: molecular cloning of a monoclonal antibody against substance P for expression in the central nervous system. *Proc Natl Acad Sci USA* **88**:5611-5615.

Rankin W, Murphy B, Grill V, Ho PWM, Moseley JM, Martin TJ (1995). Monoclonal antibody against parathyroid hormone-related protein with in vitro neutralizing activity. *Bone* **16**:205S.

Rankin W, Grill V, Martin TJ (1997). Parathyroid hormone-related protein and hypercalcemia. *Cancer* **80**: 1564-1571.

Restifo NP, Ying H, Hwang L, Leitner WW (2000). The promise of nucleic acid vaccines. *Gene Ther* **7**:89-92.

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988). Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinol* **122**:1373-1382.

Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA (1999). TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* **103**:197-206.

## Figure Legends:

*Figure 1.* Amino acid sequences of K1 and K10 light chains and the single heavy chain sequences deduced from cDNA sequences determined from PCR products cloned into pBluescript vector.

*Figure 2.* Schematic description of pVR1030 vector, indicating sites of insertion of cloned light and heavy chain segments.

*Figures 3 & 4.* Secretion of mouse IgG from transiently transfected cells. pVR3F5-K1 and -K10 DNAs were transiently transfected into 293 cells. 24 hrs later cells were switched to serum-free medium, which was removed at 72 hrs and tested by ELISA. Wells were incubated with serum-free conditioned media, washed, blocked, and then assayed for captured mouse IgG with goat anti-mouse alkaline phosphatase conjugate. A standard curve was constructed using serial dilutions of commercial mouse IgG. Results are expressed as ng/ml per 1 million cells per 48 hrs.

*Figures 5-8.* IgG from serum free conditioned media (tested in Figure 4) were concentrated by binding to Protein A/G, elution at low pH, and neutralization. IgG concentration was determined as in Figures 3 & 4. Purified IgGs were added to mouse bone marrow culture assays (Takahashi et al., 1988) stimulated with PTHrP or calcitriol. Cultures were fixed and stained for TRAP with a Sigma kit. Wells (n=4) were counted for TRAP+ cells with greater than 2 nuclei per cell. Data are expressed with standard deviations.

*Figure 9.* Concentrated K1 and K10 IgGs were tested for their ability to block PTHrP-stimulated  $\text{Ca}^{45}$  release from labeled fetal rat long bones (n=4) in culture by standard means (Garrett et al., 1990).

## **APPENDIX:**

### **1) Key research milestones:**

- Heavy chain of 3F5 monoclonal antibody cloned by PCR from hybridoma mRNA and sequenced.
- Kappa light chain (2 N-terminal polymorphic variants) cloned and sequenced, after elimination of large numbers of clones derived from aberrant light chain transcripts
- Secretory leader sequences added to two light chain cDNAs
- Light and heavy chains sequentially subcloned into pVR1030 bicistronic expression vector
- pVR1030/3F5 transiently expressed in 293 cells, resulting in secretion of mouse IgG1 protein.
- Secreted recombinant IgG neutralized PTHrP-stimulated  $\text{Ca}^{++}$  release from fetal rat long bones in organ culture
- Recombinant IgG blocked PTHrP-stimulated osteoclast formation stimulated by PTHrP in mouse bone marrow culture in vitro.
- Co-transfection of PC3 cells with pVR1030/3F5 and neo-resistance DNA resulted in initial clones secreting neutralizing antibody, when selected with the neo derivative G418. However, high level expression was not stably maintained, since the pVR1030/3F5 and neo-resistance DNAs were not physically linked to one another.

### **2) Reportable Outcomes:**

- Abstract describing initial work to be presented at 22<sup>nd</sup> annual meeting of the American

Society of Bone and Mineral Research September 25<sup>th</sup>, 2000, Toronto, Canada: Abstract # 1209: **Paracrine Neutralization of Tumor-Produced PTHrP by Bicistronic Expression of Cloned Antibody Chains.** XH Sun, X Li, WA

Rankin, BG Grubbs, V Grill, TJ Martin, GR Mundy, M Gillespie, PM Hobart, TA Guise, JM Chirgwin. University of Texas Health Science Center at San

Antonio, Peking Union Medical College Hospital, Beijing PRC, St Vincent's Institute of Medical Research, Melbourne Aus, and Vical, Inc, San Diego

- Complete sequence of clinically useful monoclonal antibody known to neutralize PTHrP determined. Light and heavy chain cDNA sequences to be deposited with Genbank when manuscript is submitted

### 3) Copies of Published Abstract (attached)

## M472

**Paracrine Neutralization of Tumor-Produced PTHrP by Bicistronic Expression of Cloned Antibody Chains.** X. Sun,<sup>1</sup> X. Li,<sup>2</sup> W. A. Rankin,<sup>1</sup> B. G. Grubbs,<sup>1</sup> V. Grill,<sup>3</sup> T. J. Martin,<sup>3</sup> G. R. Mundy,<sup>1</sup> M. T. Gillespie,<sup>3</sup> P. M. Hoban,<sup>4</sup> T. A. Quise,<sup>1</sup> J. M. Chirgwin.<sup>1</sup> <sup>1</sup>Medicine/Endocrinology, University of Texas Health Science Center, San Antonio, TX, USA. <sup>2</sup>Stomatology, Peking Union Medical College Hospital, Beijing, China. <sup>3</sup>St Vincent's Institute of Medical Research, Melbourne, Australia. <sup>4</sup>Vical, Inc. San Diego, CA, USA.

PTHrP is a major causal agent of osteolysis due to tumor cells metastatic to bone. Bone destruction can be abrogated with neutralizing antibodies, such as the mouse monoclonal antibody 3F5, which recognizes the amino terminus of PTHrP. Experiments to suppress osteolytic metastases *in vivo* require repeated systemic administration of purified antibody, and it is uncertain what treatment regimen is optimal. Since the action of PTHrP on osteolysis is known to be locally mediated, we developed a method to deliver high local concentrations of neutralizing antibody at metastatic sites. The light and heavy chains encoding anti-PTHrP mouse IgG were isolated by RT-PCR from the 3F5 hybridoma, using conserved flanking sequence primers incorporating restriction enzyme recognition sites to facilitate the subsequent cloning and expression. The IgG heavy chain was isolated, cloned, and sequenced without difficulty. The light chain cloning approach identified large numbers of aberrant transcripts, previously reported to derive from the fusion partner cell line used in hybridoma formation. A PCR primer was designed to distinguish the aberrant clones, from among over 50 of which two productive light chain cDNAs were identified and sequenced. The two light chains, designated K1 and K10, were identical except for a 3 amino acid polymorphism near the N-terminus. The two light chains, with addition of signal peptide coding sequences, and then the heavy chain, were sequentially subcloned into a bicistronic expression vector, pVR1030, which permits equivalent expression of both IgG subunits from the same plasmid, under control of a constitutive CMV promoter. When both variants (K1 and K10) of the DNA were transiently transfected into human 293 cells, the conditioned media contained mouse IgG. Serum-free conditioned media were purified on Protein A/G agarose. The eluted Ig was assayed in fetal rat long bone cultures. Both K1 and K10 IgGs, at 2 µg/ml, completely inhibited [45Ca] release in response to 10ng/ml PTHrP 1-34. We anticipate that cell lines [such as MDA-MB-231 breast cancer cells] stably expressing the bicistronic DNA encoding the 3F5 mAb will show complete neutralization of PTHrP-stimulated osteolysis.

## M473

**Parathyroid Hormone-Related Protein Inhibits Platelet-Derived Growth Factor Directed Migration of Vascular Smooth Muscle Cells in Association with Impairment of PI3 Kinase Signaling.** P. K. Kherra,\* T. Nakayama,\* T. L. Clemens. Internal Medicine, Division of Endocrinology, University of Cincinnati, Cincinnati, OH, USA.

Parathyroid hormone related protein (PTHrP) is a potent vasorelaxant and inhibitor of vascular smooth muscle cell (VSMC) growth. PTHrP expression is upregulated in neointimal VSMC following experimental arterial injury suggesting that the protein functions to oppose proliferative and migratory cues in the injured vessel wall. In this study we investigated the effects of PTHrP on A-10 VSMC migration *in vitro* using a modified Boyden chamber method. Platelet-derived growth factor (PDGF)-directed migration of serum-deprived quiescent A10 VSMC cells was maximal at four hours. PTHrP-(1-34) treatment for 30 minutes dose-dependently inhibited migration without affecting cell attachment whereas PTHrP fragments lacking the PTH-like N-terminal region including PTHrP-(38-94) and PTHrP-(67-86) had no effect. The inhibitory effect of PTHrP on cell migration was mimicked by treatment with either dibutyryl cAMP or forskolin. As PDGF is known to activate both MAP kinase PI3 kinase and MAP kinase pathways, we determined effect of the PI3 kinase and MAP kinase inhibitors on PDGF-directed migration. Treatment of cells with the PI3 kinase inhibitors wortmannin (1 µM) or LY294002 (10 µM) significantly inhibited migration whereas the Erk inhibitors PD98059 (30 µM) or U0126 (20 µM) had no effect. To examine the signal transduction events associated with PDGF-induced A10 cell migration, cell lysates from PDGF-treated cells were immunoblotted with antibodies to total Akt or phospho-Akt. PDGF-stimulated phosphorylation of Akt was inhibited by pretreatment with the PI3 kinase inhibitors wortmannin and LY294002 but not by the Erk inhibitor U0126. Treatment of cells with either 10 nM PTHrP or 2 mM db-cAMP also inhibited phosphorylation of Akt. We conclude that PTHrP, by activating adenyl cyclase, inhibits PDGF-directed VSMC migration in part by attenuating the PDGF stimulation of PI3 kinase.

## M474

**Intracrine Signaling by PTHrP Regulates a Complex Pattern of Growth-affecting Genes in Prostate Cancer.** W. Wachsmann,\* A. Gujral,\* D. Burton,\* L. J. Deftos. Department of Medicine and Cancer Centers, UCSD & SDVAMC, San Diego, CA, USA.

PTHrP (parathyroid hormone-related protein) influences the growth and proliferation of essentially every tissue in which it is expressed, both normal and malignant. Depending upon cell type and PTHrP species, the growth effect can be positive or negative. Furthermore, these effects can be mediated by the PTHrP receptor as well as by intracrine pathways that regulate cell apoptosis and proliferation. Despite the robust growth-regulating effects of PTHrP, little is known about the molecular mechanisms involved. Furthermore, only a few of the genes and their signaling pathways which are regulated by PTHrP have been identified.

We report here the use of GeneChip high-density oligonucleotide-based arrays to characterize the biological effects of PTHrP on gene expression in prostate cancer cells. The PPC-1 prostate carcinoma cell line was stably transfected with either PTHrP 1-87 or the vector control. Expression of the PTHrP in these cells was measured by epitope specific PTHrP radioimmunoassays that can identify exclusively the transfected form of the polypeptide. PTHrP concentrations were 7-fold higher in the PTHrP 1-87 line than in the vector control line.

To perform GeneChip analysis, PTHrP- and vector-transfected cells were plated and grown for 16h in normal growth medium + 5% FBS, synchronized by serum starvation for 6h, and then grown in normal growth medium + 5% FBS for 18h. Total RNA from each line was used to generate a biotinylated cRNA probe. GeneChips were hybridized with the probe for 16h, washed and stained with streptavidin-phycoerythrin, and then scanned. We then comparatively analyzed the RNA expression data obtained from the two cell preparations, using a 3-fold difference as a cut-off value. Corresponding analyses were made in other cell lines with other PTHrP species.

Our results showed that PTHrP regulates expression of scores of genes, many of which are involved in growth regulation, apoptosis, angiogenesis, and oncogenesis. Genes induced by PTHrP in prostate cancer cell lines include vascular endothelial growth factor (VEGF-B), prostate specific antigen (PSA), transforming growth factor-β 3 (TGF-β 3), L-Myc, and insulin-like growth factor 2 (IGF-2), while decreased expression was observed for p53 and bcl-xL. Our findings demonstrate the protean actions of PTHrP on gene expression in prostate cancer.

Our studies demonstrate that microarray technology has the power to identify thousands of genes in elucidating the complex molecular pathways that mediate the effects of PTHrP on the cellular biology and pathobiology of prostate cancer.

## M475

**Differential Localisation of PTHrP Functional Fragments within the Epidermis.** D. T. McCreavy,<sup>1</sup> J. A. Gallagher,\*<sup>1</sup> G. R. Sharpe,\*<sup>1</sup> W. D. Fraser.<sup>2</sup> <sup>1</sup>Human Anatomy & Cell Biology, University of Liverpool, United Kingdom. <sup>2</sup>Clinical Chemistry, University of Liverpool, United Kingdom.

A number of studies have implicated parathyroid hormone-related protein (PTHrP) in the regulation of proliferation and differentiation of keratinocytes both *in vitro* and in PTHrP knock-out mice models. As the PTHrP primary sequence contains a number of dibasic residues, known to represent substrates for prohormone convertases, it is believed that bioactive fragments are liberated during post-translational modification in a tissue specific manner. In order to establish whether specific PTHrP fragments are present within the epidermis and if so, are associated with differentiation, we have undertaken immunohistochemical studies of consecutive serial sections of normal human skin, using a panel of previously characterised antisera which bind to PTHrP (1-34), PTHrP (43-52), PTHrP (127-138) and PTHrP (145-153) fragments. We have repeatedly observed a pattern of staining using anti-PTHrP (43-52) in which a moderate signal is present within the middle layers of the stratum spinosum and this progressively increases in intensity throughout the upper layers of this strata and into the stratum granulosum. Intense staining has also been observed in isolated keratinocytes within stratum spinosum and the stratum granulosum using both anti-PTHrP (1-34) and anti-PTHrP (145-153) antisera. These observations suggest that endogenous PTHrP molecules are processed within keratinocytes of the epidermis giving rise to multiple fragments, one of which contains the PTHrP (43-52) sequence. This fragment is distributed in a manner consistent with the progressive differentiation of keratinocytes. Fragments containing PTHrP (1-34) and PTHrP (145-153) sequences are over-expressed in isolated keratinocytes. The significance of this remains to be established.

**A**

10	20	30	40	50	60
<u>METDTLLLWV LLLWVPRSTG</u> DTMETHSPSS LGVSVGEKVT MSCKSSQSLL YSYNQKNYLA					
70	80	90	100	110	120
WYQQKPGQSP KLLIYWASTR ESGVPDRFTG SGSGTDFTLT ISSVKSEDLA VYYCQQYYSY					
130	140	150	160	170	180
PYTFGGGTKL EIKRADAPT VSIFPPSSEQ LTSGGASVVC FLNNFYPKDI NVKWKIDGSE					
190	200	210	220	230	240
RQNGVLNSWT DQDSKDYTS MSSTLTITKD EYERHNSYTC EATHKTSTSP IVKSFNRNEC					

**B**

10	20	30	40	50	60
<u>METDTLLLWV LLLWVPRSTG</u> DTMESQTPSS LGVSVGEKVT MSCKSSQSLL YSYNQKNYLA					
70	80	90	100	110	120
WYQQKPGQSP KLLIYWASTR ESGVPDRFTG SGSGTDFTLT ISSVKSEDLA VYYCQQYYSY					
130	140	150	160	170	180
PYTFGGGTKL EIKRADAPT VSIFPPSSEQ LTSGGASVVC FLNNFYPKDI NVKWKIDGSE					
190	200	210	220	230	240
RQNGVLNSWT DQDSKDYTS MSSTLTITKD EYERHNSYTC EATHKTSTSP IVKSFNRNEC					

**C**

10	20	30	40	50	60
<u>MDFGLSLVFL VLVLKGVQCE</u> VMLVESGGGL VKPGGSLKLS CAASGFTFSN YAMSWVRQTP					
70	80	90	100	110	120
EKRLEWVATI TSGDTYTYYP DSVKGRFTIS RDNAKNTLYL QMSSLRSEDY AIYYCARRVI					
130	140	150	160	170	180
RPVDYASDYW GQGTSVTVSS AKTTPPSVYP LAPGSAAQTN SMVTLGCLVK GYFPEPVTVT					
190	200	210	220	230	240
WNSGSLSSGV HTPPAVLQSD LYTLSSSVTV PSSTWPSETV TCNVAHPASS TKVDKKIVPR					
250	260	270	280	290	300
DCGCKPCICT VPEVSSVFIF PPKPKDVLTI TLTPKVTCVV VDISKDDPEV QFSWFVDDVE					
310	320	330	340	350	360
VHTAQTQPRE EQFNSTFRSV SELPIMHQDW LNGKEFKCRV NSAAFPAPIE KTISKTKGRP					
370	380	390	400	410	420
KAPQVYTIPP PKEQMAKDKV SLTCMITDFF PEDITVEWQW NGQPAENYKN TQPIMDTDGS					
430	440	450	460		
YFVYSKLNQV KSNWEAGNTF TCSVLHEGLH NHHTEKSLSH SPGK					

**FIGURE 1.** Amino acid sequences of the cloned light and heavy chains of 3F5 mAb. **A**, kappa light chain of K1 clone; **B**, kappa light chain of K10 clone; **C**, heavy chain. In **A** and **B**, the first 22 amino acids prior to Met23 are added synthetically to encode secretory signal peptide. Signal peptides are underlined in all sequences. K1 and K10 sequences [**A** and **B**] differ only at residues 25-27.

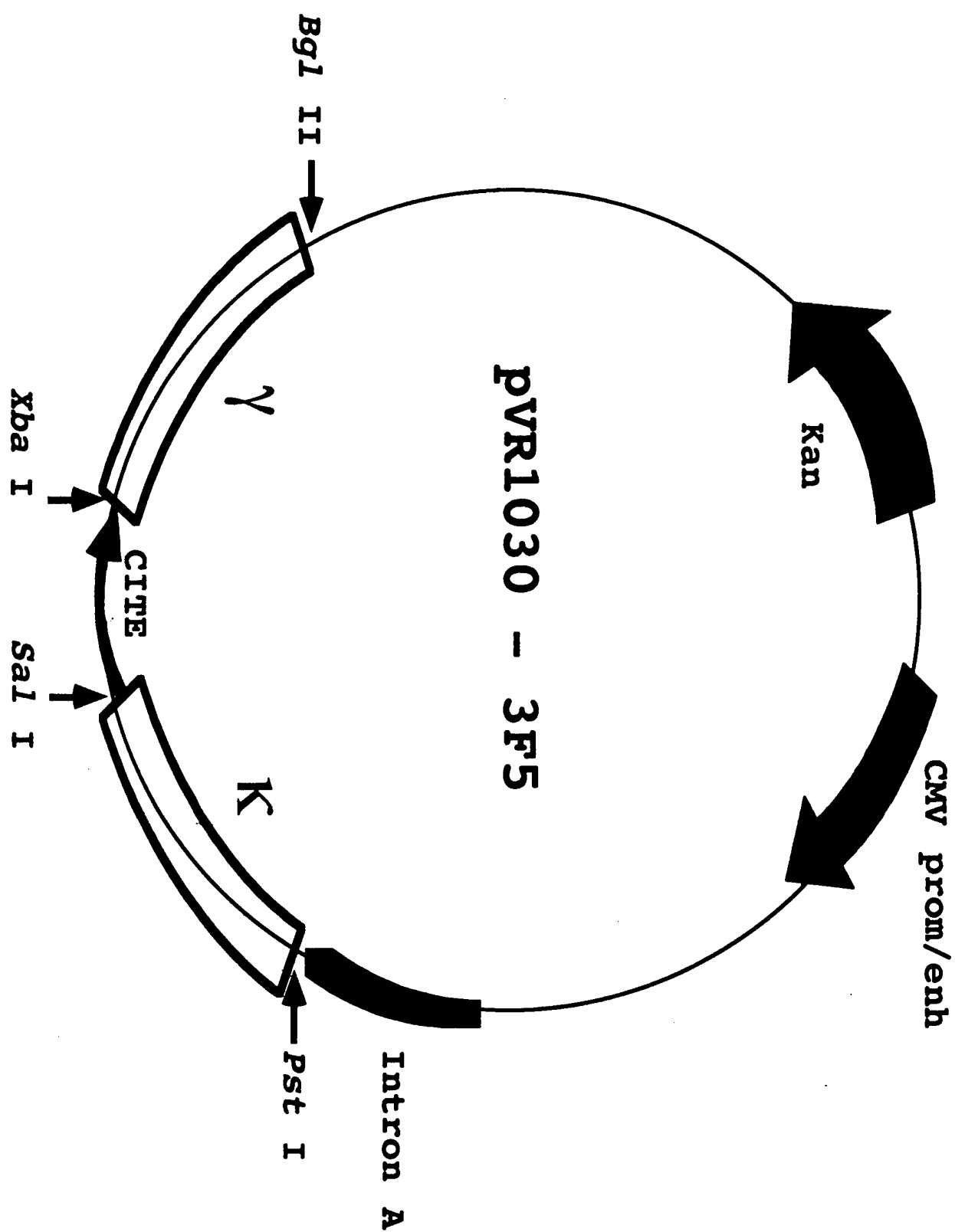


Figure 2



# ELISA of CM of 3F5 Transient Transfection 293 cells in 6 well plates

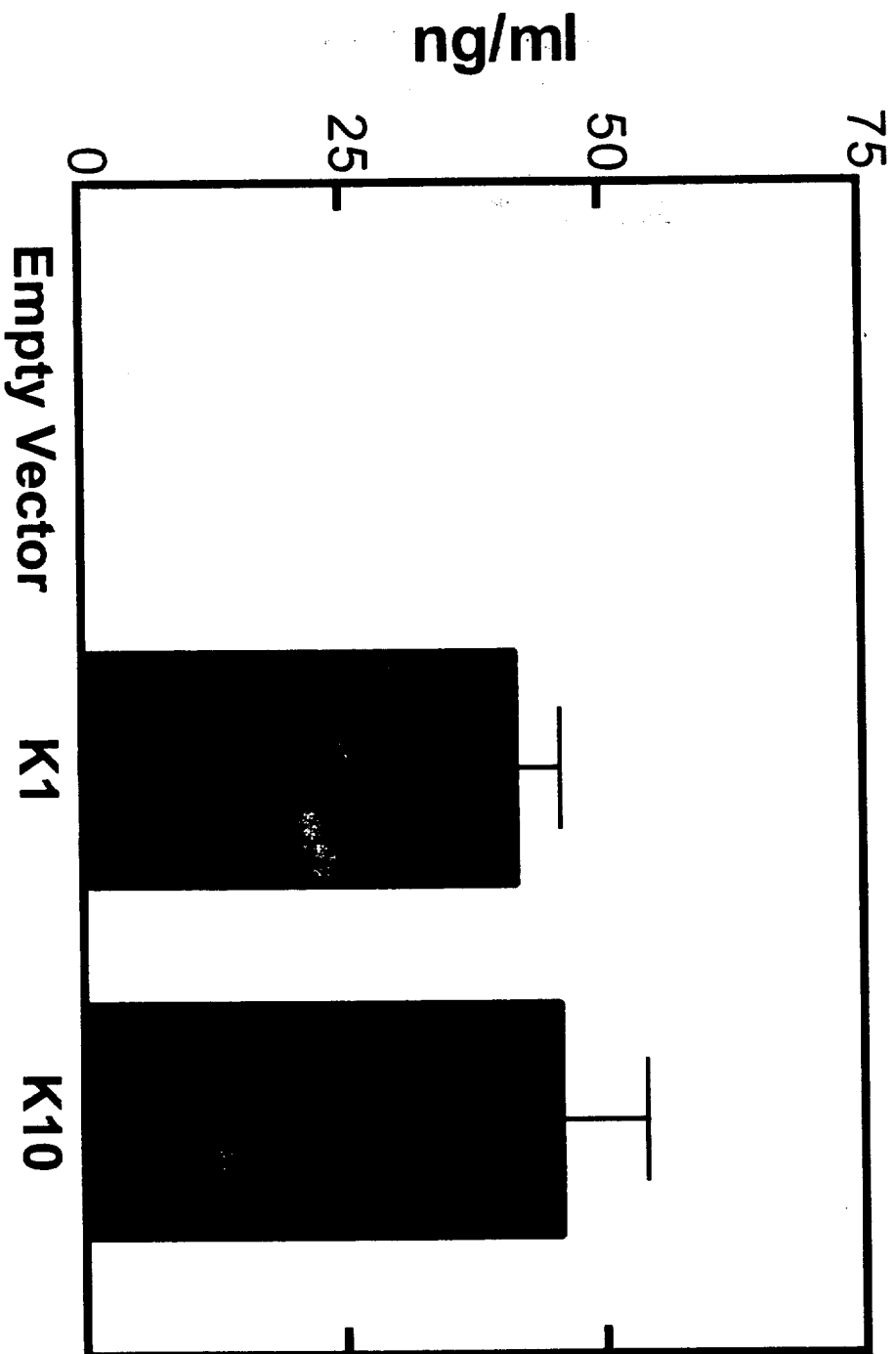


Figure 3

# ELISA of CM of 3F5 Transient Transfection 293 cells in T150 flask

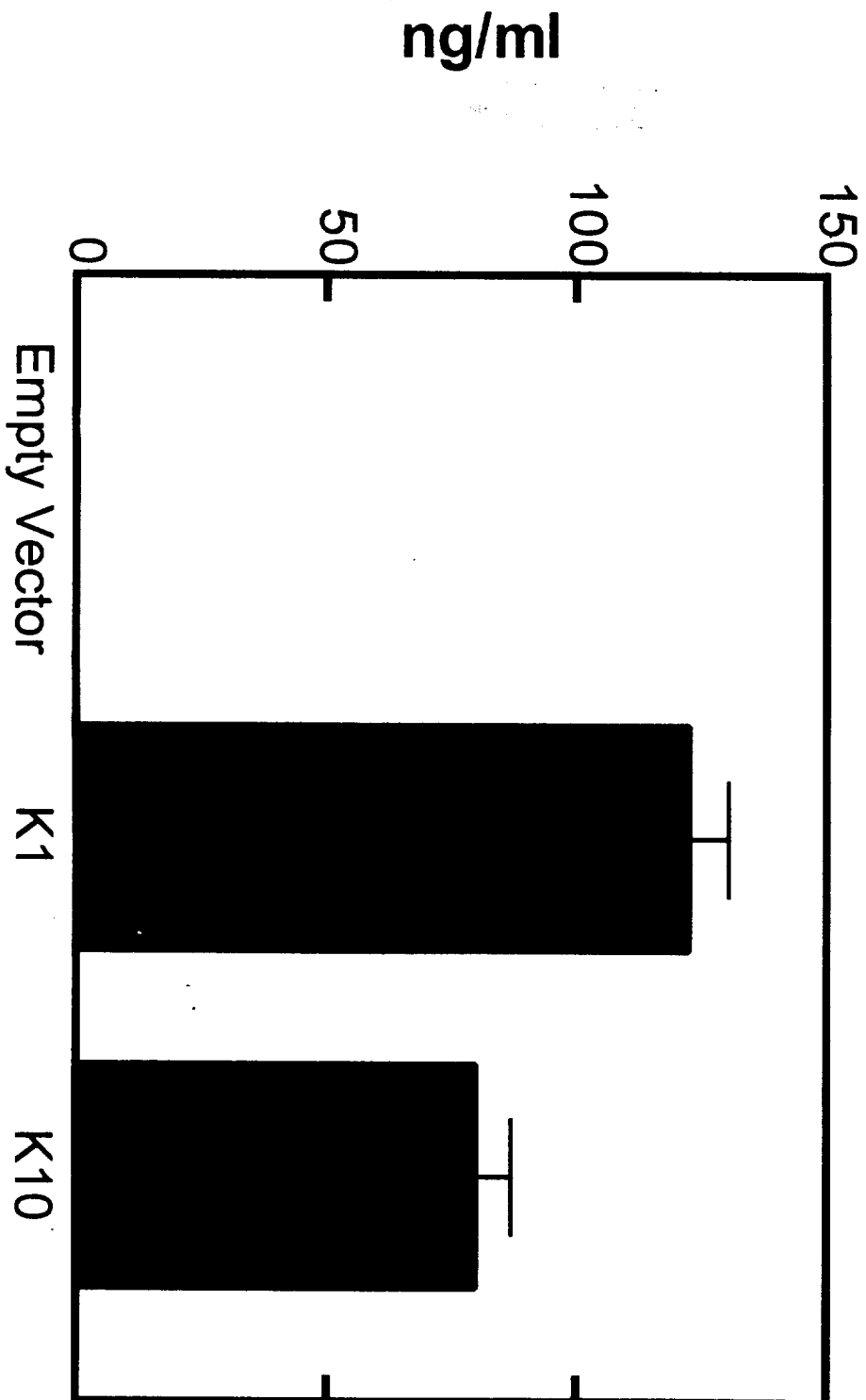


Figure 4

# Mouse Bone Marrow Assay

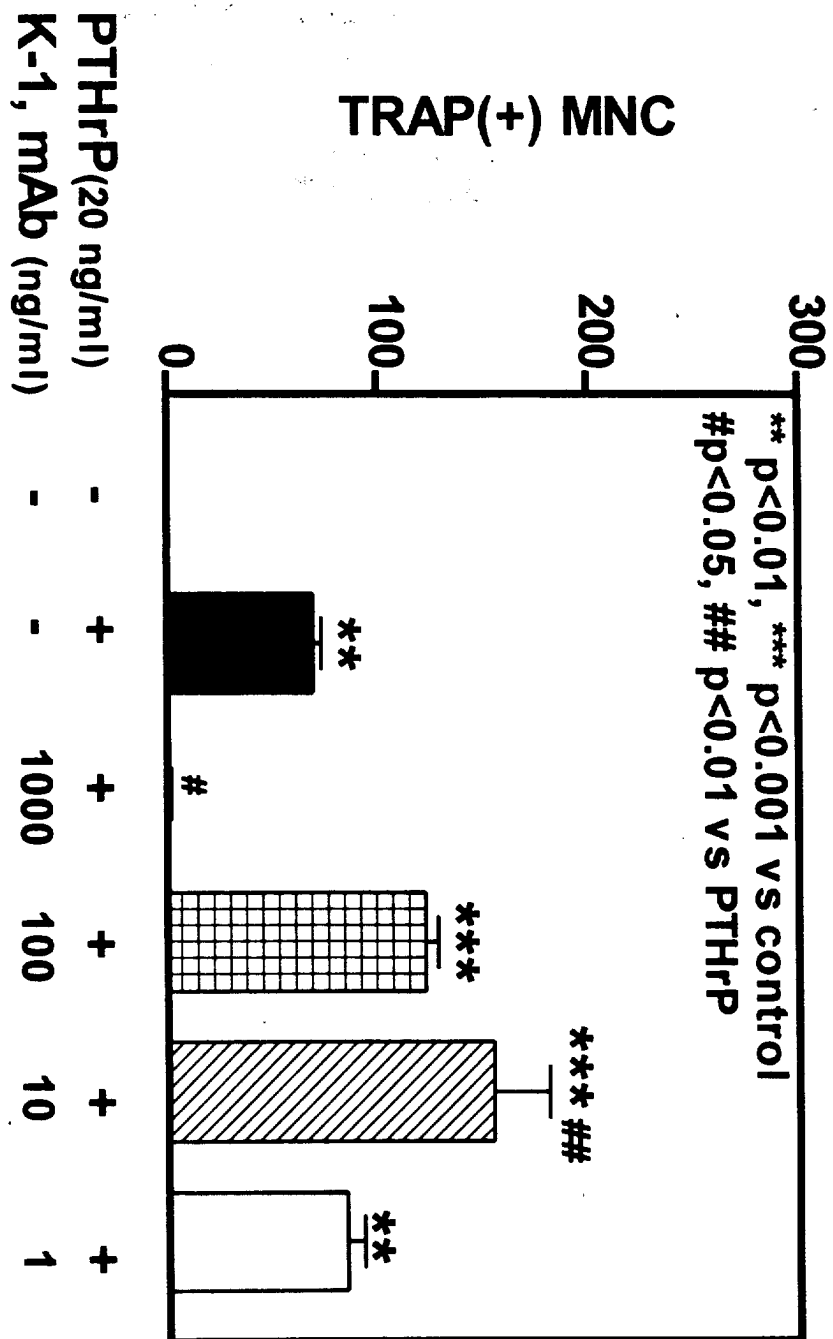


Figure 5

# Mouse Bone Marrow Assay

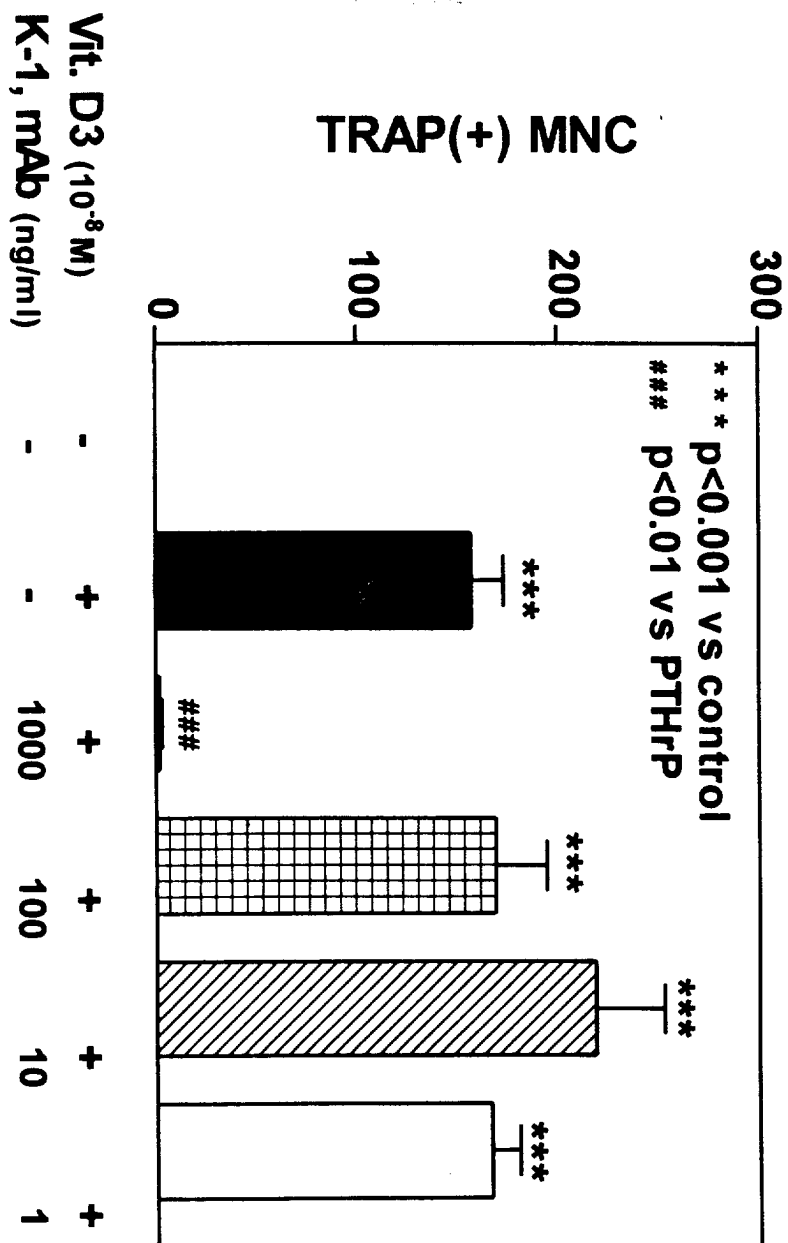


Figure 6

# Mouse Bone Marrow Assay

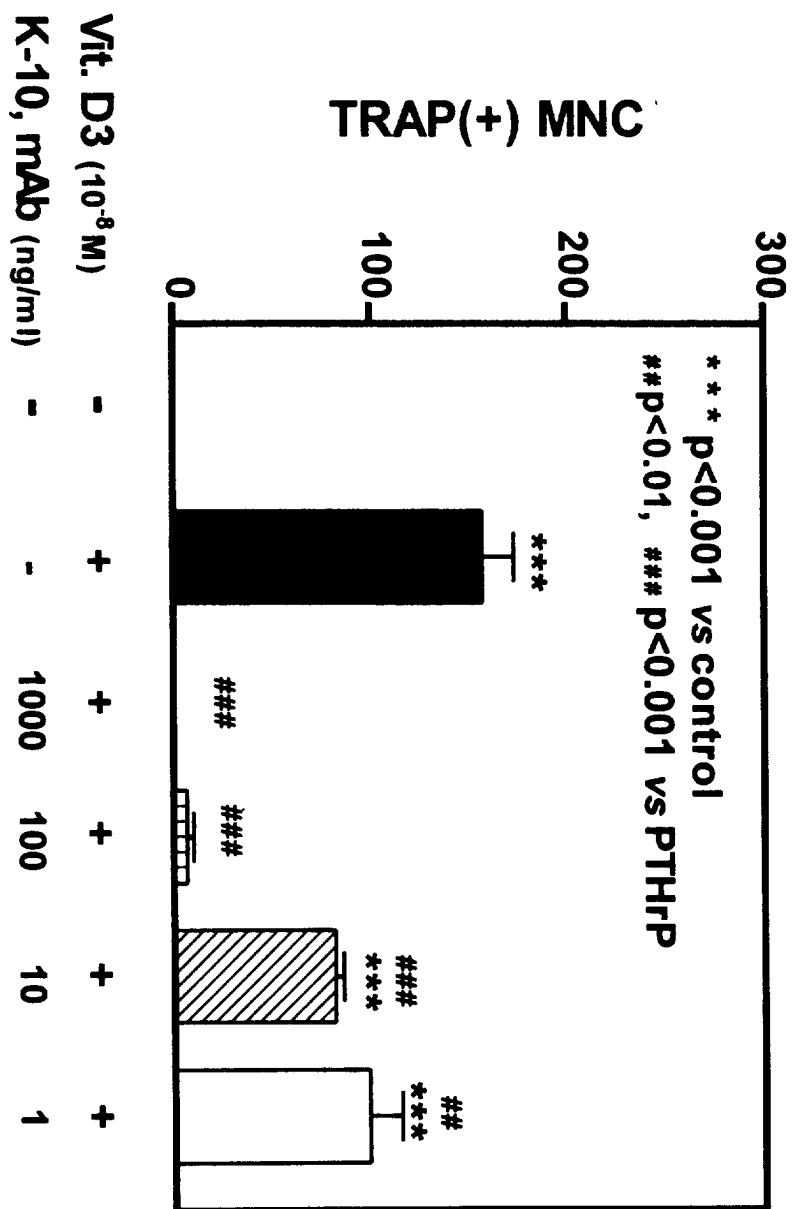


Figure 7

# Mouse Bone Marrow Assay

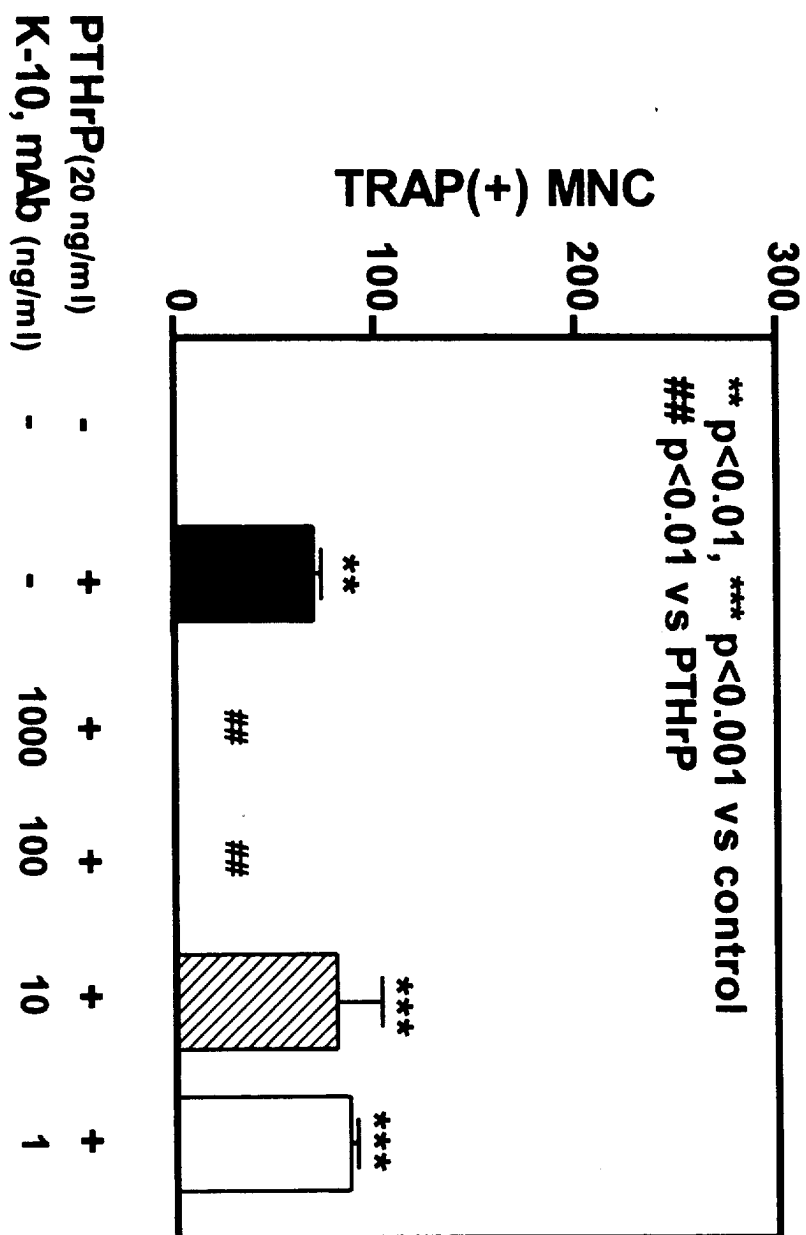


Figure 8

# Fetal Rat Long Bone Assay

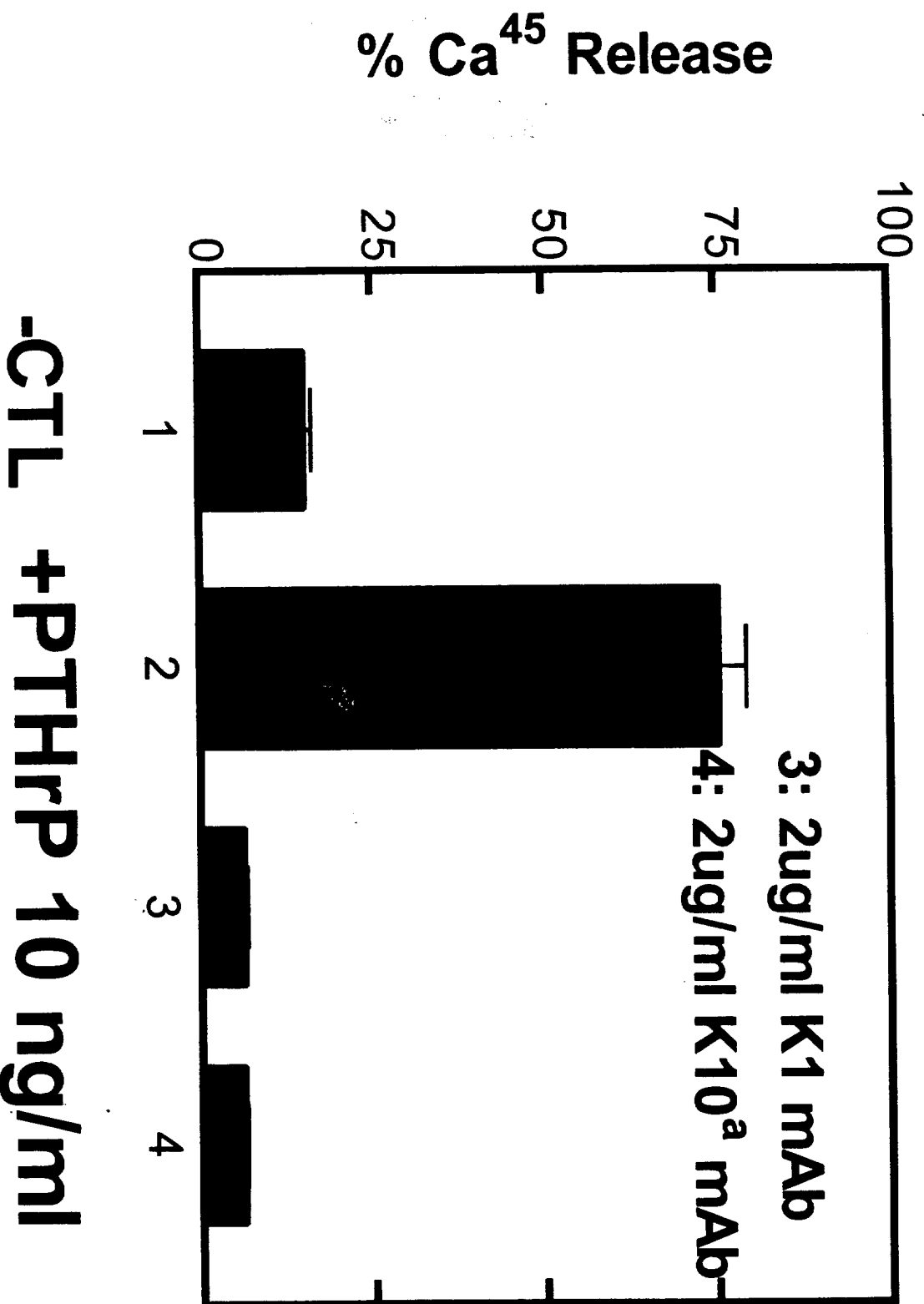


Figure 9

## HYBRIDOMA FUSION CELL LINES CONTAIN AN ABERRANT KAPPA TRANSCRIPT

WILLIAM L. CARROLL,\*† EILEEN MENDEL\* and SHOSHANA LEVY\*

Departments of Medicine\* and Pediatrics,† Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

(First received 12 January 1988; accepted in revised form 31 March 1988)

**Abstract**—The V region sequence of a non-productive kappa transcript from two myeloma fusion partners has been determined. This transcript has an aberrant VJ recombination site resulting in a translation stop site at position 105. It is variably expressed in hybridomas made from all fusion partners derived from the original MOPC-21 tumor. The amount of this transcript may greatly exceed levels of the productive light chain mRNA.

### INTRODUCTION

Analysis of immunoglobulin (Ig) genes expressed by individual B-cells during an immune response has been facilitated by expanding such single cells in the form of hybridomas. Typically, such hybridomas are used as a source not only of Ig protein but also of Ig mRNA that can be sequenced directly, or cloned and subsequently sequenced (Gearhart *et al.*, 1981; Kaartinen *et al.*, 1983; Clarke *et al.*, 1985). We have used this approach to study somatic mutations in Ig variable (V) regions of human (Cleary *et al.*, 1986) and murine B-cells tumors (Carroll *et al.*, manuscript in preparation) and have developed a rapid method for cloning and sequencing these genes (Levy *et al.*, 1987). mRNA from hybridomas of either mouse  $\times$  mouse or human  $\times$  mouse fusions is used as a template for cDNA synthesis primed by Ig constant region specific primers.

With the use of heavy chain specific primers we have consistently obtained a single  $V_H$  cDNA species from each hybridoma. However, using kappa specific primers we have often obtained two different  $V_K$  cDNA clones from each hybridoma, only one of which is derived from the B-cell. The second cDNA species arises from an aberrant mRNA transcript which is present in all standard fusion partners derived from the original MOPC-21 tumor (X63-Ag8, NS-1, P3X63Ag8.653). Here we report the sequence of this  $V_K$  region, its expression in the various hybridoma fusion lines and its enhanced transcription in a particular heterohybridoma.

This work was supported by USPHS grant CA 33399 to Dr Ronald Levy. W. L. Carroll is a recipient of an NIH clinical investigator award CA 01059. Computer resources used to carry out this study were provided by the National Institutes of Health-sponsored BIONET National Computer Research for Molecular Biology (grant number RR-01685-05).

### MATERIALS AND METHODS

#### Cell lines and hybrids

The genealogy of the myeloma fusion partners described in this report is shown in Fig. 1. Murine myelomas X63-Ag8, P3/NS-1/1-Ag4-1 (NS-1) and P3X63-Ag8.653 (8.653) were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD) and all were derived from the MOPC-21 myeloma tumor. X63-Ag8 secretes the MOPC-21 immunoglobulin IgG1( $\kappa$ ) (Kohler and Milstein, 1975), NS-1 contains intracellular MOPC-21  $\kappa$  light chain protein (Kohler and Milstein, 1976) and 8.653 produces no immunoglobulin protein (Kearney *et al.*, 1979). K6H6/B5 is a mouse  $\times$  human heteromyeloma useful for fusion with B-cells of human origin (Carroll *et al.*, 1986). It was generated by a fusion between NS-1 and a human follicular lymphoma (IgM,  $\lambda$ ) and is derived from a clone that lost all Ig secretion and was reselected for HAT sensitivity.

Somatic cell hybridizations between lymphoma cells and non-secreting myelomas were performed by standard techniques (Carroll *et al.*, 1986). Human lymphomas were fused with K6H6/B5 whereas the murine B-cell lymphoma 38C13 was fused with

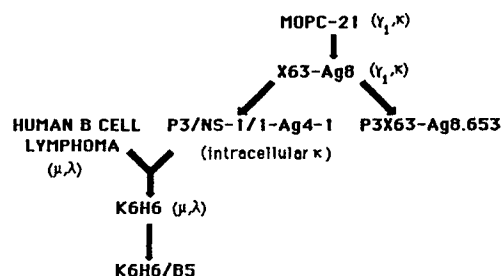


Fig. 1. Genealogy of myeloma fusion cell lines and their Ig products.



murine myeloma 8.653. The resulting hybrids were screened by ELISA for Ig secretion and were cloned by limiting dilution using irradiated BALB/C spleen cells as a feeder layer.

#### Cloning and sequencing of cDNA

cDNA libraries were made from individual hybridomas by methods described in detail previously (Levy *et al.*, 1987). The Ig V regions were cloned by specifically priming V region cDNA synthesis with oligonucleotides homologous to heavy and light chain constant regions just 3' to the variable region. Second strand cDNA was made by using a combination of DNA polymerase I and RNAaseH (Gubler and Hoffman, 1983). Double stranded cDNA was polished with T<sub>4</sub> DNA polymerase (New England Biolabs, Beverly, MA) and ligated directly into the SmaI site of M13mp19, using T<sub>4</sub> DNA ligase (New England Biolabs). To prevent religation of the vector itself, the SmaI digested M13mp19 was pretreated with alkaline phosphatase. JM101 bacteria were made competent by the Hanahan method (1983), and transformed by the recombinant M13. After transformation plaques were lifted onto nitrocellulose and were probed with either <sup>32</sup>P labeled single stranded cDNA made by primer extension or with appropriate V region genes that had been cloned previously. Positive hybridizing clones were selected for the appropriate size inserts and the V regions were sequenced using the dideoxy chain termination technique (Sanger *et al.*, 1987).

#### RNA isolation and Northern blotting

Total RNA was isolated from myelomas and hybridomas as previously described (Cleary *et al.*, 1986); poly (A)<sup>+</sup> RNA was selected by chromatography on oligo (dT) cellulose (Aviv and Leder, 1972). Total RNA or poly (A)<sup>+</sup> RNA was electrophoresed using glyoxal and dimethyl sulfoxide as denaturing agents according to Thomas (1980) or by using the formaldehyde gel technique (Lehrach *et al.*, 1977). RNA was then transferred to nitrocellulose and hybridized with <sup>32</sup>P labeled probes. A 510 b.p. EcoRI-HpaI murine constant region (C<sub>κ</sub>) kappa probe was obtained from a full length cDNA clone encoding the 38C13 B-cell lymphoma light chain sequence (Campbell *et al.*, 1987). A human C<sub>κ</sub> probe consisted of a 2.5 kb EcoRI genomic fragment encompassing the entire human C<sub>κ</sub> gene (Hieter *et al.*, 1980). A variable region cDNA probe specific for the aberrant kappa transcript was obtained by methods described above. All probes were labeled by random hexamer priming (Feinberg and Vogelstein, 1983). Hybridization and washing conditions were used as described previously (Levy *et al.*, 1987).

## RESULTS

#### Hybridomas consistently contain a non-functional kappa transcript

Hybridomas were constructed by the fusion of mouse B-cell tumor (38C13) with 8.653, hetero-hybridomas were constructed by fusing human B-cell

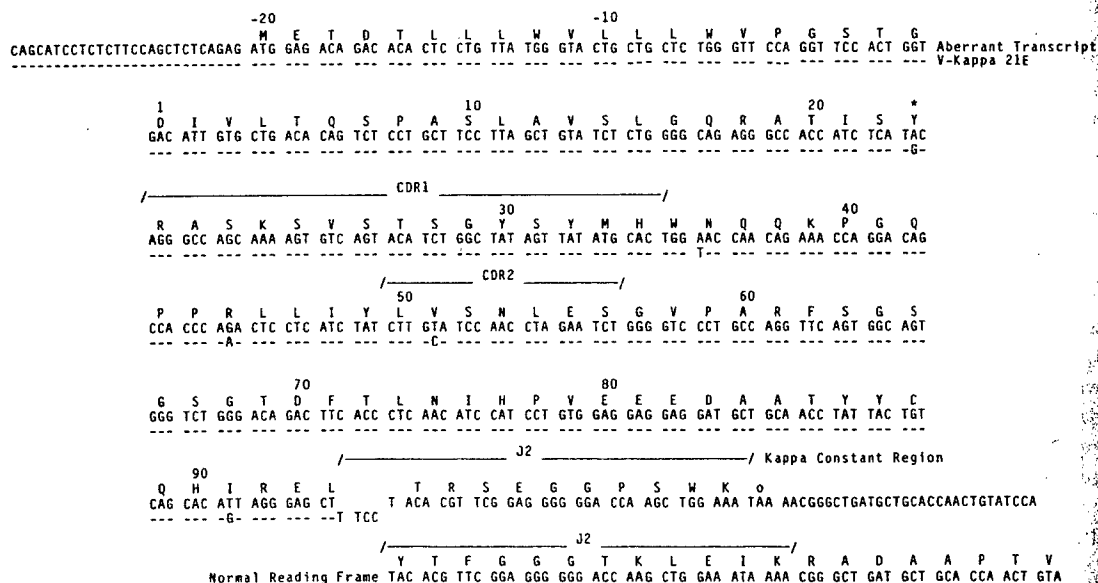


Fig. 2. Nucleotide sequence of the aberrant V<sub>κ</sub> transcript in mouse myeloma fusion partners. The amino acid translation is given above the nucleotide sequence of the aberrant transcript with numbering according to Kabat *et al.* (1987). Homologies with germ line V<sub>κ</sub> 21E are indicated by dashes and base changes are shown; the \* indicates a change that results in replacement of the invariant cysteine in position 23. The complementarity determining regions CDR 1 and CDR 2 are indicated, as is the J2 segment. The VJ joint is out of frame, resulting in a stop codon in position 105.

tumors with K6H6/B5. Messenger RNA was isolated and immunoglobulin V region cDNA was cloned using specific primers for  $\mu$  or  $\kappa$  constant regions as detailed in Materials and Methods. A single heavy chain V region sequence, the product of the B-cell tumor in each case, was identified from cDNA libraries made from individual hybridomas. However, unexpectedly, two kappa sequences were consistently seen in libraries constructed using a kappa specific primer. One encoded the productively rearranged transcript of mouse or human origin, depending on the tumor source used in the fusion. A second sequence, of murine origin and shown in Fig. 2, has been repeatedly identified in various independent hybridomas and in human  $\times$  mouse heterohybridomas. It is interesting to note that the human kappa primer was efficient in priming cDNA synthesis on the mouse kappa transcript in spite of 3/17 mismatched bases. This sequence is not the MOPC-21 chain (Hamlyn *et al.*, 1981). Rather it bears close homology to the murine  $V_{\kappa}$  21E germ line gene (Heinrich *et al.*, 1984) rearranged to the  $J_2$  segment (Kabat *et al.*, 1987). However, at the site of VJ recombination four nucleotides appear to have been deleted, leading to a frame shift. This results in a premature termination codon at position 105. In addition, the invariant cysteine in position 23 is replaced by tyrosine.

#### Expression of the aberrant kappa gene in myeloma fusion lines and hybridomas

In order to determine if a kappa transcript exists in 8.653, this line as well as its parent X63-Ag8 and NS-1 were obtained from the ATCC. The latter two lines express kappa protein and would be expected to display specific mRNA, whereas 8.653 does not produce any Ig protein chains (Kearney *et al.*, 1979). Northern blot analysis using a murine  $C_{\kappa}$  probe is shown in Fig. 3. As expected, kappa message is present in X63-Ag8 and NS-1 (lanes 1 and 2). However, a normal sized kappa transcript is identified in 8.653 as well (lane 3). A similar pattern was obtained when the same filter was probed with the  $V_{\kappa}$  region gene of the aberrant transcript (data not shown). This transcript cannot represent the productively rearranged MOPC-21 gene, since very stringent hybridization washing conditions were applied ( $63^{\circ}\text{C}$ ,  $0.1 \times \text{SSC}$ ,  $0.1\%$  SDS). A homology search between the MOPC-21  $V_{\kappa}$  gene (Kabat *et al.*, 1987) and the aberrant  $V_{\kappa}$  gene reveal only several short (8–12 b.p.) stretches of complete homology.

In order to compare the expression of the aberrant kappa gene with the functional secreted kappa, Northern blot analysis was performed on human heterohybridomas using mouse and human probes. Two such hybridomas, both derived from the same human B-cell lymphoma, were tested along with the relevant fusion partner K6H6/B5. Identical lanes were run, one set was probed with a human C $\kappa$  sequence and the other was probed with the aberrant

V region clone. As can be seen in Fig. 4, K6H6/B5 contained small amounts of the aberrant mouse transcript but no human kappa transcript (lane 1, panel A vs B). One heterohybridoma (2A12) contained large amounts of the aberrant mouse transcript and lesser amounts of the human transcript whereas the reverse was true for another heterohybridoma (1B11).

#### DISCUSSION

These experiments demonstrate the presence of an aberrant transcript in non-secreting myeloma fusion partners 8.653 and K6H6/B5. Since these two lines are derived by parallel lineages from P3X63-Ag8 (Fig. 1) the origin of this transcript would appear to be MOPC-21. However, the V sequence reported here is not the productive light chain of this myeloma (Hamlyn *et al.*, 1981) and the aberrant VJ recombination site suggest that it is a non-functional rearrangement which is nevertheless transcribed into RNA. This V region is a member of the  $V_{\kappa}$ -21 family, it is most homologous to  $V_{\kappa}$  21 E of the 11 sequenced genes of this 13 gene family (Heinrich *et al.*, 1984).

Two different kappa transcripts have been demonstrated previously in kappa producing myelomas and kappa mRNA has also been detected in  $\lambda$  producing

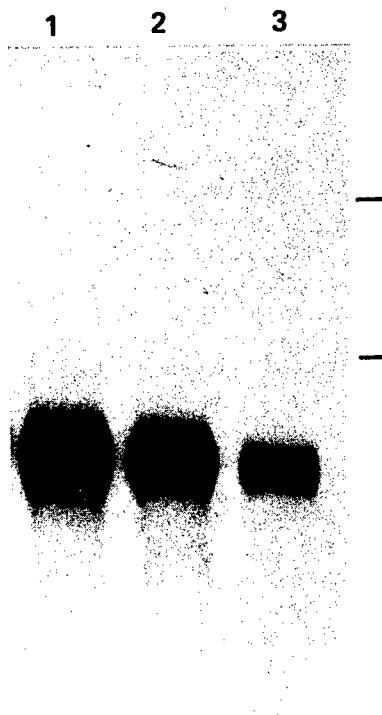


Fig. 3. Expression of the aberrant kappa transcript in mouse fusion partner lines.  $15 \mu\text{g}$  of RNA was loaded per lane and electrophoresed on a formaldehyde gel. RNA was transferred to nitrocellulose and probed with a 510 b.p.  $\kappa$  cDNA (Campbell *et al.*, 1987). Lane 1, X63-Ag8. Lane 2, NS-1. Lane 3, 8.653. Rules refer to location of 28S and 18S RNA.

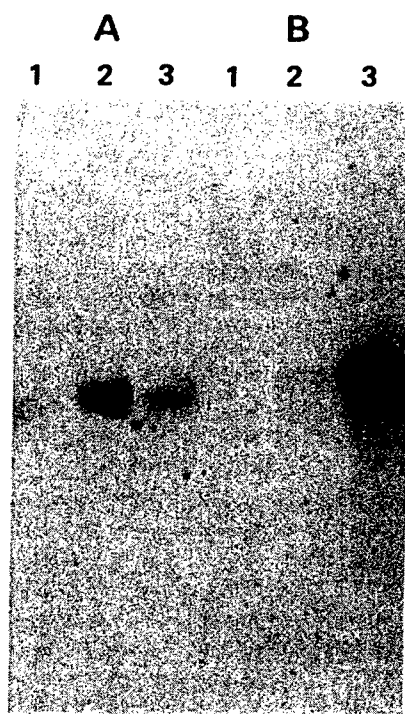


Fig. 4. Variable expression of human kappa and the aberrant mouse transcript in human  $\times$  mouse heterohybridomas. 10  $\mu$ g of RNA was loaded in each lane and electrophoresed and transferred according to Thomas (1980). The RNA blot was hybridized to the aberrant kappa transcript cDNA clone (A) and to a human C kappa probe (Hieter *et al.*, 1980) (B) RNA from the fusion partner K6H6/B5 in lane 1 and from the heterohybridomas 2A12 and 1B11 in lanes 2 and 3 respectively.

myelomas (Alt *et al.*, 1980; Kelley *et al.*, 1985; Kwan *et al.*, 1981; Perry *et al.*, 1980). Even though multiple transcripts may be present, only one allele produces the secreted light chain protein. In many cases the aberrantly rearranged transcripts produce kappa protein fragments (Alt *et al.*, 1980). The transcript reported here contains a translation termination site at codon 105, and of interest is the fact that this V region codes for tyrosine instead of the invariant cysteine at position 23. Although 8.653 does not produce intracellular kappa protein when analyzed with antisera directed against kappa constant region (Kearney *et al.*, 1979), it is possible that a V region fragment is translated in these cells.

The organization of rearranged MOPC-21 kappa genes has previously been studied (Walfield *et al.*, 1980). MOPC-21 and NS-1 contain two rearrangements, the functional one being lost in subclones of NS-1. A 127 b.p. portion of the non-functional rearranged kappa transcript in NS-1n, overlapping the VJ junction, has been sequenced and is identical to the sequence reported here with the exception of an A instead of a G at the first nucleotide of codon 84 (Walfield *et al.*, 1981). We believe that this discrepancy is likely to be due to gel reading error (A instead of G) inherent in the Maxam and Gilbert technique.

Thus, this gene is highly conserved in many commonly used fusion partners and is present in Sp2/0-Ag14 as well (Leahy *et al.*, 1988). There is no evidence for mutation in this V region gene despite prolonged growth in culture and its presence in various hybridomas using multiple different sources of B-cells.

We also demonstrate that the expression of the kappa transcript is variable. It can be quite high in certain hybridomas and may actually exceed levels of the productive light chain mRNA. Control of the level of this transcript might reside on a separate chromosome that is lost in the fusion process. The expression of this aberrant transcript can interfere with  $\kappa$  mRNA sequencing by producing ambiguity in the sequencing reactions. cDNA cloning will separate the two different transcripts. The sequence of the aberrant transcript, presented here, will allow others to distinguish it from the relevant kappa gene in such cases.

**Acknowledgements**—The authors thank Dr Ronald Levy for his support and encouragement, Dan Leahy for critically reviewing the manuscript, and Phyllis Bussey and Emily Chatfield for their assistance in preparing this manuscript.

#### REFERENCES

- Alt F. W., Enea V., Bothwell A. L. M. and Baltimore D. (1980) Activity of multiple light chain genes in murine myeloma cells producing a single, functional light chain. *Cell* **21**, 1–12.
- Aviv H. and Leder P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408–1412.
- Campbell M. J., Carroll W., Kon S., Thielemans K., Rothbard J. B., Levy S. and Levy R. (1987) Humoral and cellular responses elicited by tumor-derived IgM and its molecular subunits. *J. Immun.* **139**, 2825–2833.
- Carroll W. L., Thielemans K., Dilley J. and Levy R. (1986) Mouse  $\times$  human heterohybridomas as fusion partners with human B cell tumors. *J. Immun. Meth.* **89**, 61–72.
- Clarke S. H., Huppi K., Ruzinsky D., Staudt L., Gerhard W. and Weigert M. (1985) Inter- and intraclonal diversity in the antibody response to influenza hemagglutinin. *J. exp. Med.* **161**, 687–704.
- Cleary M. L., Meeker T. C., Levy S., Lee E., Trela M., Sklar J. and Levy R. (1986) Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. *Cell* **44**, 97–106.
- Feinberg A. D. and Vogelstein B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13.
- Gearhart P. J., Johnson N. D., Douglas R. and Hood L. (1981) IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. *Nature* **291**, 29–34.
- Gubler U. and Hoffman B. J. (1983) A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263–269.
- Hamlyn P. H., Gait M. J. and Milstein C. (1981) Complete sequence of an immunoglobulin mRNA using specific priming and the dideoxynucleotide method of RNA sequencing. *Nucl. Acids Res.* **9**, 4485–4494.
- Hanahan D. (1983) Studies on transformation of *E. coli* with plasmids. *J. Molec. Biol.* **166**, 557–580.

- Heinrich G., Traunecker A. and Tonegawa S. (1984) Somatic mutation creates diversity in the major group of mouse immunoglobulin  $\kappa$  light chains. *J. exp. Med.* **159**, 417-435.
- Hieter P. A., Max E. E., Seidman J. G., Maizel J. V. and Leder P. (1980) Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. *Cell* **22**, 197-207.
- Kaartinen M., Griffiths G. M., Markham N. F. and Milstein C. (1983) mRNA sequences define an unusually restricted IgG response to a  $\alpha$ -phenyloxazalone and its early diversification. *Nature* **304**, 320-324.
- Kabat E. A., Wu T. T., Reid-Miller M., Perry H. M. and Gottsman K. S. (1987) Sequences of proteins of immunological interest. (Bethesda, Maryland: U.S. Public Health Service.)
- Kearney J. G., Radbruch A., Liesegang B. and Rajewsky K. (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immun.* **123**, 1548-1550.
- Kelley D. E., Wiedemann L. M., Pittet A.-C., Strauss S., Nelson K. J., Davis J., Van Ness B. and Perry R. P. (1985) Nonproductive kappa immunoglobulin genes: recombinational abnormalities and other lesions affecting transcription, RNA processing, turnover, and translation. *Molec. Cell. Biol.* **5**, 1660-1675.
- Kohler G. and Milstein C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497.
- Kohler G. and Milstein C. (1976) Derivation of specific antibody producing tissue culture and tumor lines by cell fusion. *Eur. J. Immun.* **6**, 511-519.
- Kwan S.-P., Max E. E., Seidman J. G., Leder P. and Scharff M. D. (1981) Two kappa immunoglobulin genes are expressed in the myeloma S107. *Cell* **26**, 57-66.
- Leahy D. J., Rule G. S., Whittaker M. M. and McConnell H. M. DNA sequences of twelve monoclonal anti-spin label antibodies for NMR studies. *Proc. natn. Acad. Sci.* (in press).
- Lehrach H., Diamond D., Wozney J. M. and Boedtker H. (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* **16**, 4743-4751.
- Levy S., Mendel E. and Kon S. (1987) A rapid method for cloning and sequencing expressed immunoglobulin variable region genes. *Gene* **54**, 165-173.
- Perry R. P., Kelley D. E., Coleclough C., Seidman J. G., Leder P., Tonegawa S., Matthysens G. and Weigert M. (1980) Transcription of mouse K chain genes: Implications for allelic exclusion. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1937-1941.
- Sanger F., Nicklen S. and Coulson A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467.
- Thomas P. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205.
- Walfield A., Selsing E., Arp B. and Storb U. (1981) Misalignment of V and J gene segments resulting in a nonfunctional immunoglobulin gene. *Nucl. Acids Res.* **9**, 1101-1109.
- Walfield A. M., Storb U., Selsing E. and Zentgraf H. (1980) Comparison of different rearranged immunoglobulin kappa genes of a myeloma by electronmicroscopy and restriction mapping of cloned DNA: implications for "allelic exclusion". *Nucl. Acids Res.* **8**, 4689-4707.